The endogenous thrombin potential

Challenges in Medicine

Herausforderungen der Medizin
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Ausblick

Präsynaptische Autoinhibition und Kotransmission sind Funktionsprinzipien des Sympathikus. Wie hätte sich Hans Castorps Herz erst gebärdet, wäre die Freisetzung von Noradrenalin nicht durch α_2- (und vielleicht P_2-) Autoinhibition gebremst worden (Abb. 1)? Sicher trug zur Entglutung seines Gesichts neben adrenerger auch purinerge Vasokonstriktion bei (Abb. 2). Eine lebendige Wissenschaft beantwortet Fragen und stellt Fragen: Welche Rolle Autoinhibition und Kotransmission bei der Informationsübertragung vom Sympathikus auf die Mm. arrectores pilorum spielen, so daß ihn Schauer überliefen, das liegt im dunkeln.

Im Winter vor jenem Faschingsdienstag liest Hans Castorp im Liegestuhl auf seinem Balkon Werke der Anatomie, Physiologie und Lebenskunde (und nur noch selten „Ocean steamships"). Er sinnt:

„Was war das Leben? Man wußte es nicht. Es war sich seiner bewußt, unzweifelhaft, sobald es Leben war, aber es wußte nicht, was es sei. ... Niemand verstand den Stoffwechsel, niemand das Wesen der Nervenfunktion. ... was hatte es auf sich mit der weißen und grauen Substanz des Kopfmarks, was mit dem Sehhügel, der mit dem Optikus kommunizierte, und mit den grauen Einlagerungen der ‚Brücke'? Die Hirn- und Rückenmarksubstanz war dermaßen zersetzlich, daß keine Hoffnung bestand, je ihren Aufbau zu ergründen. Was enthob beim Einschlafen die Großhirnrinde ihrer Tätigkeit? ... Aber was bedeutete all dieses Unwissen im Vergleich mit der Ratlosigkeit, in der man vor Erscheinungen wie der des Gedächtnisses ... stand?"

Wenn „Wesen" Metaphysisches meint, kann Naturwissenschaft in der Tat das „Wesen der Nervenfunktion" nicht verstehen. Aber manches interessante Detail kann sie enthüllen. 1924 war die chemische Natur der nervalen Informationsübertragung schon seit drei Jahren entdeckt, und zwar an den parasympathischen Nerven zum Herzen. Nach langem Streit wurde sie auch für Gehirn und Rückenmark anerkannt. Autoinhibition und Kotransmission sind demgegenüber Prinzipien niedrigeren Ranges. Doch haben auch sie unser Bild der nervalen Informationsübertragung im Sympathikus, im übrigen peripheren Nervensystem und im Zentralnervensystem merklich verändert (eine allgemeine Übersicht bei Starke 1996).

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The Endogenous Thrombin Potential, a New Laboratory Parameter to Indicate the Risk of Thrombosis and its Diminution by Treatment

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Ernst Jung-Prize for Medicine 1985

Prof. Hemker, given the Prize in 1985, is a specialist in haemostasis and thrombosis with a great international reputation. He was able to develop efficient methods to quantify and describe the factors in blood plasma which condition haemostatic processes. To Prof. Hemker we owe a new understanding of the function of vitamin K and the key role of lipids and platelets when it comes to haemostasis.

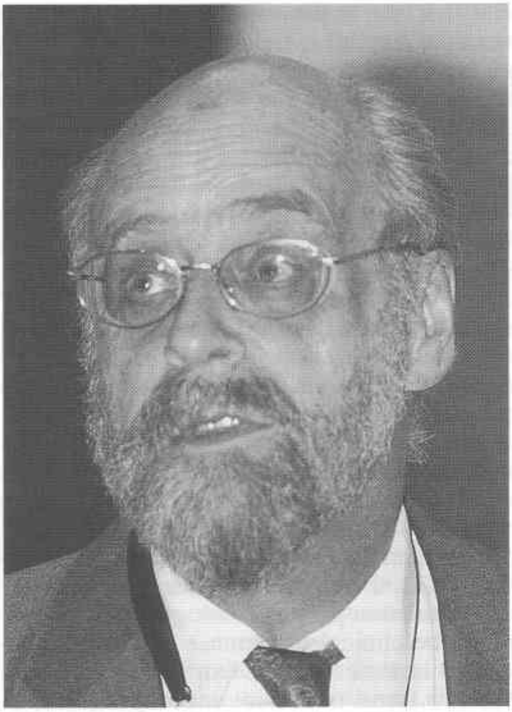
One can no longer imagine treating hypertension without measuring blood pressure or investigating arrhythmia without the electrocardiogram. As a matter of course we require tests that make sure that blood is pumped round at the right pressure and in the right rhythm. Still we hardly realise that we do not dispose of a simple test to probe the system that makes that there is any liquid at all to be pumped round: the blood coagulation system. Yet, coronary infarction, stroke, pulmonary embolism and at least half a dozen of less common but equally dangerous and disabling diseases are simply caused by the fact that blood stops to be liquid, so that no further flow is possible, with disastrous consequences for the tissues downstream; in other word: thrombosis. Thrombosis is at the basis of more than half of all mortality in the western world and still it remains somewhat of a stepchild in circulation research. Until recently it was hardly recognised that it is the main pathogenic mechanism behind acute cardiovascular disease. Haemostasis and blood coagulation were considered to an area remote to the interest of the cardiologist and almost incomprehensible anyhow.

Decreasing the coagulability of the blood, i.e. the administration of anticoagulant drugs, can substantially decrease the incidence of thrombosis. It carries the risk of bleeding and therefore should be carefully controlled. One of the most serious handicaps in the fight against thrombosis is that we do not know who is going to get thrombosis and therefore should receive anticoagulation. Another problem is that the control of anticoagulation is a complicated and specialistic affair. If one and the same test could first be used for detection of a thrombotic tendency and then show how this tendency decreases upon anticoagulation, then a major step forward would have been made. In other words, we need a test, that, like blood pressure in hypertension is a direct measure of the relevant parameter and directly reflects the result of pharmacological intervention. This article reports on the results of our quest for such a test. At the moment we are in the exciting stage where, on good grounds, we think that we have found one, but the conclusive proof can only be given by thorough and extensive clinical testing, which is still lacking.

To present our results, it is not necessary to discuss the haemostatic mechanism in detail. The important thing to know is that blood, upon contact with anything else than the normal endothelial lining of the blood vessel will start to generate thrombin via a complicated interplay of plasmatic proteins, blood platelets and the triggering components from perivascular tissue. Thrombin is the active agent that brings about haemostasis as well as thrombosis. It also engages in a set of positive and negative feedback reactions that ensure its explosive generation at –, and limitation to –, the site of vascular injury. Its enzymatic action on plasma (coagulation), blood platelets (aggregation and activation of procoagulant activities) and on the cells of the vessel wall (smooth muscle cells, fibroblasts endothelial cells) that work together to achieve arrest of blood-flow locally. If a wound triggers this process, the mechanism serves the vital function of haemostasis, i.e. the prevention of blood loss. It is however also possible that degenerative (atherosclerotic) changes in the vessel wall are the trigger of this process, in that case thrombosis ensues. This does not necessarily lead to an immediate dramatic result, as long as it does not completely occlude an important vessel. Given the fact however than non-occlusive thrombosis is an important step in the progression of atherosclerotic disease, it always is a step in the direction of a possible dramatic ending.

Recent research, among others from the group of Fuster, leaves little doubt that thrombus formation on a (ruptured) atherosclerotic plaque is the main cause of coronary infarction. There is no reason to assume that in other organs (e.g. brain) this would be different. All in all it is evident that the capacity of blood to form thrombin is the key pathophysiological function that we need to modulate in order to prevent and treat thrombosis and that, therefore, we should be able to measure. The idea to measure the thrombin that is generated in clotting blood is older than the century but until now the experimental procedures were extremely cumbersome and there was no accurate and simple test that could be used in clinical practice.

If a sample of blood is taken and some tissue extract is added to mimic contact with extravascular tissue, then thrombin is generated, the concentration of which can be determined in subsamples taken from the mixture. The life-time of thrombin is limited because plasma not only contains a (quick) thrombin generating mechanism but also a (slower) thrombin inhibiting system. This explains why the concentration of thrombin first



Professor Dr. med. H. Coenraad Hemker

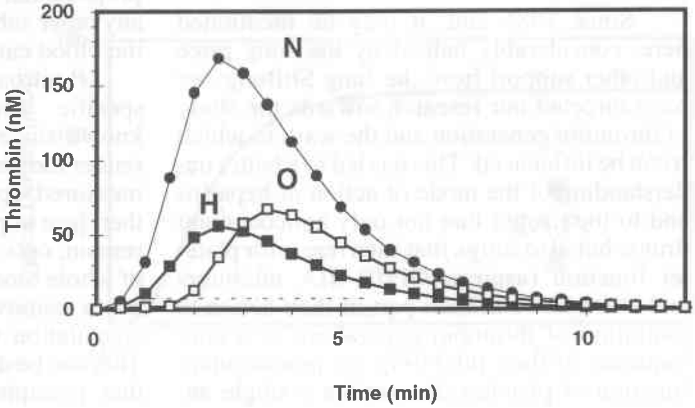


Fig. 1 Thrombin Generation Curves
Blood is brought to clot outside the body by adding tissue factor and calcium ions. Every half minute the concentration of thrombin is measured in a subsample. Clotting occurs when a thrombin level of 10 nM is reached (horizontal dotted line), i.e. early in the process. The normal curve rises to over 150 nM. In anticoagulation the peak value is considerably lower. Heparin Induced anticoagulation (H) is not accompanied by a significant increase of the clotting time, in contrast to oral anticoagulation (O)

rises (as long as the rate of formation is higher than the rate of breakdown) and then falls (as soon as breakdown gains over generation), to reach a zero level eventually (fig.1). The actual clotting of the blood occurs as soon as 5–10 nM of thrombin are formed but thrombin generation continues until a peak of about 200 nM is reached. This means that the bulk of thrombin is generated *after* clotting has taken place. This is the main reason that the clotting *time* does not necessarily reflect the *amount* of thrombin that will form. The haemostatic and thrombotic action of thrombin is a consequence of its enzymatic action on various physiological substrates (fibrinogen, cell receptors etc.). The extent of this action is determined by the amount of thrombin and by the time that it can act, i.e. to the area under the thrombin – time curve (fig.1). This area, that we called it the Endogenous Thrombin Potential (ETP), therefore is an essential parameter in coagulation pathophysiology.

The clinical importance of the ETP becomes immediately evident if one considers the two forms of current anticoagulant medication: vitamin K antagonists and heparin. The administration of vitamin K antagonists decreases the concentration of thrombin-forming proteins in the blood plasma; consequently less thrombin can generate and the ETP diminishes. In constrast, heparin (at clinically applied doses) leaves the thrombin generation system essentially unaffected but diminishes the area under the thrombin – time curve by accelerating thrombin breakdown. Although their mode of action is completely different and the only thing they have in common is that they both diminish the ETP, both treatments are effective in preventing thrombosis.

Since 1985 and, it may be mentioned here, considerably helped by the Jung price and other support from the Jung Stiftung, we have directed our research towards the study of thrombin generation and the ways in which it can be influenced. This has led to a better understanding of the mode of action of heparins and to the insight that not only anticoagulant drugs, but also drugs that interfere with platelet function (aspirin, GPIIB-IIIA inhibitors and others) owe at least part of their action to inhibition of thrombin generation, as a consequence of their inhibiting the procoagulant function of platelets. In fact not a single antithrombotic that we tested would *not* inhibit thrombin generation in blood! Moreover it appeared that the ETP is *increased* in prothrombotic conditiions, such as the use of oral contraceptives and congenital deficiencies of antithrombin and proteins C or S. So it seems that the ETP rises in prothrombotic states and lowers upon antithrombotic medication. Clotting assays do not indicate prothrombotic states and may or may not vary with antithrombotic medication, depending on the type of test and the type of drug used. The bleeding time, apart from being impossible to standardise, only prolongs with certain antiplatelet drugs (aspirin) and is not shortened in patients with a thrombotic tendency. It thus appeared that the ETP would be a valuable asset for the routine clinical laboratory.

Our early observations on the ETP were done with the subsampling technique which requires taking 20–40 samples in the course of 15 minutes and determination of the thrombin concentration therein. This test is unsuitable for clinical and epidemiological research, given the hundreds of determinations that may be required per day. We therefore set out to find a more practical method.

In principle, the solution is exceedingly simple. One only needs to add a suitable thrombin substrate to a blood sample and to measure the amount of product formed during the coagulation process. The ideal substrate should be converted by thrombin only; should render an easily detectable product; and should not be depleted during coagulation. In fact the concentration of substrate should be so high that only variations of the concentration of thrombin determine the amount of product formed, which amount by definition is proportional to the ETP, i.e. to the amount of any other substrate that thrombin generated in the blood can convert.

P-nitroanilide-oligopeptides that are specific thrombin-substrates have been known since a long time. As a product they render the yellow *p*-nitroaniline, which can be measured spectrophotometrically. Their use therefore imposes that the system must be, and remain, optically clear. This precludes the use of whole blood. Plasma must be pre-treated so as to remove the fibrinogen, which upon coagulation would render the sample turbid. This can be done with a snake venom protease that precipitates fibrinogen but leaves the thrombin generating- and inhibiting system intact.

The existing chromogenic substrates for thrombin are unsuitable for our purposes, because they are split too quickly and exhaust during the coagulation process. We therefore screened a large number of different p-nitroanilides, several dozens of which we synthesised ourselves. We did find a few suitable ones. When such a substrate is added to plasma and the optical density is monitored then a curve such as in fig.2 should be obtained. At any moment the velocity of colour formation is proportional to the concentration of thrombin. The first derivative of the curve therefore gives the course of thrombin generation (as in fig.1) and the amount of product formed in the end is proportional to the ETP (fig.2).

Mind however that we said "should be observed". In practice a complication arises from the fact that thrombin partakes in a side-reaction. With the plasma protein α_2-macroglobulin it forms a complex that has no activity with (large) physiological thrombin substrates but that still is able to split (small) oligopeptide substrates, such as the signalling peptide added. This makes that a composite curve is obtained that consists of two parts: the product formed by thrombin itself and the product formed by the α2-macroglobulin-thrombin complex. Because the velocity of complex formation in itself is proportional with the concentration of thrombin, the latter part of the curve is proportional to the integral of the former. We will not go into this into more detail here. Suffice it to say that it is possible, with the aid of a relatively simple numeric calculation procedure (that can be carried out by every personal computer), to split the composite signal into the two partial curves (fig.3). The ETP then is proportional to the end-level of product that is converted by free thrombin. It would be ideal to have a chromogenic substrate that is not converted by α2-macroglobulin-thrombin, but up to this moment we did not find one.

Does this end-level indeed always represent the real ETP? There is at least one caveat: the thrombin generating process involves several enzymes that resemble thrombin closely. They may therefore possibly interact with the signalling substrate that is added. Due to their low concentrations this will not cause a significant increase of product formation but the substrate might inhibit thrombin formation by competition with the natural processes. Such inhibiting substrates were discarded as not suitable. Competition is inevitable, however, between the added substrate and the natural thrombin inhibitors of plasma, because inhibitors can only attack thrombin that is not bound to the substrate. As we know the binding constant (Michaelis constant, Km) of the enzyme-substrate interaction, we can calculate what proportion of thrombin is bound at a given substrate concentration (S/(Km+S)). Inded it could be verified that the normal thrombin decay processes were decreased in that proportion, so that correction by a factor (Km+S)/S gives the required value.

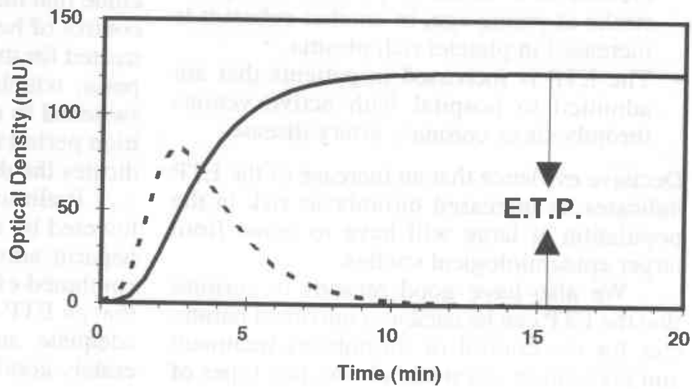


Fig. 2 Conversion of a signal substrate by thrombin generated in clotting plasma.
A chromogenic substrate is added to clotting plasma in high enough concentration for the reaction velocity to be proportional to the thrombin concentration. Product formation is monitored via the optical density (solid line). The first derivative of this curve (dotted line) then is proportional to the thrombin generation curve (cf. fig. 1). The end-level of product is proportional to the endogenous thrombin potential.

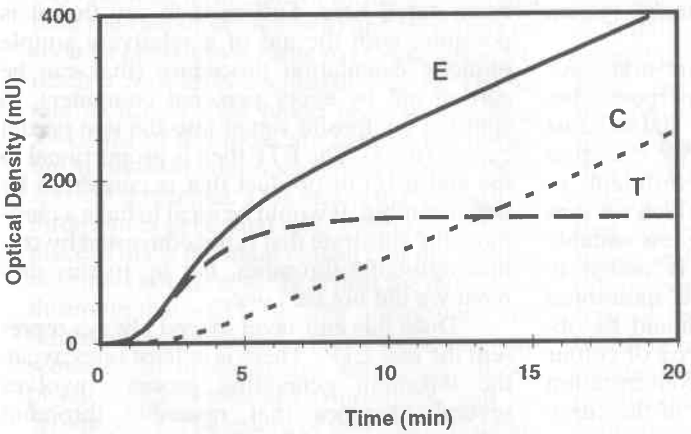


Fig. 3 The E. T. P. determination in practice.
In practice a complex forms between a part of the free thrombin that is generated and alpha-2 macroglobulin. This complex has an enzymatic activity towards the chromogenic substrate. The optical density signal that is obtained (line E) therefore is not that which would be theoretically expected (cf. fig. 2). By a mathematical procedure curve E can be split into the part due to free thrombin (T, i. e. the curve as in fig. 2) and the part due to the complex (C, proportional to the integral of T). The end level of T gives the E. T. P.

If the last two paragraphs may appear slightly technical, we still think that they are necessary, were it only to indicate that we can account for the disturbances that we bring about by probing the system.

The ETP-determination now being available we may well ask what the existing evidence is that it can be used to screen for thrombosis prone states and to monitor the effect of antithrombotic drugs.

The suggestive evidence that a high ETP indicates a thrombotic risk is the following:

- The ETP is increased in women using oral contraceptives
- The ETP is increased in congenital deficiencies of the anticoagulant proteins C and S as well as antithrombin.
- The ETP in platelet poor plasma is increased in a subset of patients that got a stroke at young age, in another subset it is increased in platelet rich plasma.
- The ETP is increased in patients that are admitted to hospital with active venous thrombosis or coronary artery disease.

Decisive evidence that an increase of the ETP indicates an increased thrombosis risk in the population at large will have to come from larger epidemiological studies.

We also have good reasons to surmise that the ETP can be used as a universal parameter for the control of thrombosis treatment and prevention. As stated above, two types of pharmaca are used for this purpose: oral administration of vitamin K antagonists or injection of heparin. Oral anticoagulant is present at monitored by the prothrombin time. Since the different types of this test have been made comparable by the introduction of the international normalised ration (INR) this works reasonably well in practice. The prothrombin time is insensitive to heparin however. For this drug the activated partial thromboplastin time (APTT) is used. This test is very method-dependent, almost impossible to standardise and relatively insensitive; even less sensitive to low molecular weight heparins than to unfractionated (classical) heparin. It therefore does not surprise that in several studies no correlations are found between the outcome of the APTT and rethrombosis or bleeding in patients that receive heparin. We must conclude that there is no adequate method for the control of heparin treatment. Patients that are treated for thrombosis usually first receive heparin, which acts immediately and then are switched to oral anticoagulation. In the transition period there is no test that adequately indicates the degree of anticoagulation.

Preliminary studies show that the ETP is lowered by oral anticoagulation as well as by heparin administration. It also indicates the combined effect of mixed treatment. It seems that an ETP of between 10 and 20% indicates adequate anticoagulation. There is a moderately good correlation between the ETP and the outcome of PT and APTT tests ($r^2 = 0.6-0.7$). The interesting question is whether the ETP or the classical test is the better indicator of bleeding or rethrombosis. This question can only be solved by larger clinical trials.

In conclusion, we may say that we have developed a test that, if it keeps its promises, may profoundly change the prevention and treatment of thromboembolic disease. The proof of the pudding will, as always, remain in the eating.

Further Reading

In a publication of this nature it is hardly useful to give extensive references to the literature. We therefore only mention a couple of articles for futher reading and in which complete references can be found.

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